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(57) Abstract

Vectors containing a sufficient number of nucleotides to express HIV gene products necessary to be replication-competent and also containing an ELI *nef* gene segment are disclosed. These vectors can be used to transfect a wide range of cells and hosts for use in assay systems.

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EXPRESSION VECTORS CONTAINING THE HIV *nef* GENE TO ENHANCE REPLICATION EFFICIENCY

The present invention is directed to a vector comprising a replication-competent HIV-1 provirus or HIV-2 provirus and a sequence which enhances the replication efficiency of said provirus.

The human immunodeficiency virus (HIV-I, also referred to as HTLV-III, LAV or HTLV-III/LAV) is the etiological agent of the acquired immune deficiency syndrome (AIDS) and related disorders [Barre-Sinoussi, et al., Science 220:868-871 (1983); Gallo et al, Science 224:500-503 (1984); Levy et al., Science 225;840-842 (1984); Popovic et al., Science 224:497-500 (1984); Sarngadharan et al., Science 224:506-508 (1984); Siegal et al., N. Engl. J. Med. 305:1439-1444 (1981)]. The disease is characterized by a long asymptomatic period followed by progressive degeneration of the immune system and the central nervous system. Studies of the virus indicate that replication is highly regulated, and both latent and lytic infection of the CD4 positive helper subset of T-lymphocytes occur in tissue culture [Zagury et al., Science 231:850-853 (1986)]. The expression of the virus in infected patients also appears to be regulated to enable evasion of the immune response. Molecular studies of the regulation and genomic organization of HIV-I show that it encodes a number of genes [Ratner et al., Nature 313:277-284

(1985); Sanchez-Pescador et al., Science 227:484-492 (1985); Muesing et al., Nature 313:450-457 (1985); Wain-Hobson et al., Cell 40:9-17 (1985)].

Retroviruses are typically classified as belonging to one of three subfamilies, namely oncoviruses, spumaviruses and lentiviruses. Infection by oncoviruses is typically associated with malignant disorders. These viruses typically contain a single-stranded, plus-strand RNA genome of approximately 8,000 to 10,000 nucleotides encompassing the gag, pol and env, genes, as well as long terminal repeat (LTR) sequences. Oncoviruses typically contain an oncogene. It is generally believed that spumaviruses are not pathogenic in vivo, although they induce foamy cytopathic changes in tissue culture. Infection by lentiviruses is generally slow and causes chronic debilitating diseases after a long latency period. Lentiviruses, in addition to the gag, pol, and env genes possess a number of additional genes with regulatory functions.

The human immunodeficiency viruses (HIV) has been classified as a lentivirus, because it too causes slow infection and has structural properties in common with such viruses. [See Haase, A.T., Nature 322:130-136 (1986)].

HIV-1 shares the gag, pol and env genes, respectively with the other retroviruses [Haseltine, W.A., Journal of Acquired Immune Deficiency Syndrome, 1:217-240 (1988)]. HIV-1 also possesses additional genes modulating viral replication. The HIV-1 genome encodes vif, vpr, tat, rev, vpu and nef proteins [Haseltine, W.A., Journal of Acquired Immune Deficiency Syndrome, supra]. Additionally, the long terminal repeats (LTRs) of HIV contain cis-acting sequences

that are important for integration, transcription and polyadenylation. Additional cis-acting signals allow regulation of HIV sequences by some of the novel HIV gene products [Haseltine, W.A., Journal of Acquired Immune Deficiency Syndrome, supra; Sodroski et al., Science 231:1549-1553 (1986); Arya et al., Science 229:69-73 (1985); Sodroski et al., Science 227:171-173 (1985); Sodroski et al., Nature 321:412-417 (1986); Feinberg et al., Cell 46:807-817 (1986) Wong-Staal et al, AIDS Res. and Human Retroviruses 3:33-39 (1987); which are all incorporated herein by reference].

Nucleotide sequences from HIV-2 and simian immunodeficiency virus (SIV) also contain the structural genes, gag, pol, and env as well as regulatory sequences such as tat, rev, and nef [Guyader, M., et al., Nature, 326:662-669 (1987); Chakrabarti, L., et al., Nature 328:543-547 (1987), which are incorporated herein by reference].

The nef gene of the primate immunodeficiency viruses, HIV-1, HIV-2 and SIV, distinguish these viruses from other retroviruses. The ungulate lentiviruses, such as the Visna virus, caprine arthritis, and encephalitis virus (CAEV) and the equine infectious anemia virus (EIAV), which are the retroviruses most closely related to these primate immunodeficiency viruses, lack the region which specifies nef, namely the long open-reading frame that begins near the 3' end of the envelope (env) and extends into the 3' LTR.

The nef protein specified by the HIV and SIV viruses share common features, including post-translational modification by addition of the fatty acid, myristic acid at the amino terminus and regions of conserved amino acid

sequences. The protein encoded by the HIV-1 nef gene is typically 206 amino acid long, although some variations occur depending upon the strain. Furthermore, antibodies to nef protein are found in infected individuals and in infected monkeys. This would suggest that nef plays an important role in natural infection. However, this is contradicted by other data. For example, the nef protein is prematurely truncated in many replication-competent HIV-1 isolates.

Initial studies using proviruses derived from the IIIB strain of HIV-1 revealed that viruses that express nef protein replicate slightly more slowly in $CD4^+$ T cell cultures than do viruses defective for nef [Terwilliger, E., et al. J. Virol. 60:754-760 (1986) which is incorporated herein by reference]. This finding has been confirmed using several other pairs of viruses, including an isogenic pair derived from the BRU and NY5 strains [Luciw, P.A., et al., PNAS 84:1434-1438 (1987); Niederman, T.M., et al., PNAS 86:1128-1132 (1989); Ahmad, N., et al., Science 241:1481-1485 (1988); and Cheng-Myer, C., et al., Science 246:1629-1632 (1989), which are all incorporated herein by reference]. Differences of 5 to 10 fold in replication of nef^+ as compared to nef^- viruses have been reported for growth of these viruses in T-cells. It is also reported that $CD4^+$T cell lines that constitutively express nef, slow the growth of many strains of HIV-1 [Cheng, et al., Science 246, supra]. This difference between nef^+ and nef^- viruses, however, is not always found. In some studies, nef positive viruses replicated as well or slightly better than nef^- defective viruses.

Although a great deal of research has been expended on understanding the human immunodeficiency viruses

(particularly, HIV-1 and HIV-2), there have been problems with fully understanding their life cycle. Individuals infected with these viruses typically exhibit a lengthy asymptomatic period during which viral titers are low and levels of $T4^+$ lymphocytes are normal, and such a phase typically extends over a period of years. The virus also does not cause infection in most animals. While chimpanzees can be infected, it is very difficult to follow the viruses in chimpanzees, as they do not readily display signs of infection and very little viral protein or RNA is made.

Accordingly, it would be extremely useful to have a vector containing nucleotides corresponding to a replication-competent HIV provirus and a sequence which makes it easier to cause infection in a wider range of cells and a wider range of animals with the provirus in order to be able to develop animal models for treating the disease.

In particular, it would be useful to use such a vector in a system where the virus can be followed in vitro and in vivo in a wide range of cells to screen for drugs that inhibit infection and to be able to develop drugs that might stop or minimize infections in certain cells.

It would also be useful to use such a vector in a system where the virus can be followed in vivo in developing a viable animal model for studying different aspects of HIV pathogenesis.

It would also be useful to have a more aggressive virus, which would provide a more visible target for an animal's immune system, to provoke a more intense and effective immune response in order to develop a vaccine and/or antibodies.

Summary of the Invention

We have now developed a vector comprising a sufficient number of nucleotides corresponding to an HIV genome, other than all structural genes of the ELI strain, to express HIV gene products necessary for viral replication and infectivity (HIV segment) and also containing a sequence of nucleotides corresponding to the nef gene of the ELI strain of HIV (nef gene segment). Preferably, the HIV segment of this vector would also contain a functional vpu gene segment or a functional vpr gene segment. Still more preferably, the HIV segment of this vector would contain both a functional vpu and a functional vpr gene segment.

In a second embodiment we describe a vector comprising an ELI gene segment. Preferably, this vector also contains a heterologous gene.

The vectors can be used in vivo and in vitro to transfect a wide variety of cell lines.

Brief Description of the Drawings

Figure 1 is a schematic diagram of an HIV genome and a series of vectors prepared.

Figure 2 is a schematic diagram of a portion of the HXB ELI 1-fs vectors.

Figure 3 is a schematic diagram of a portion of the HXB ELI 2 and HXB ELI 2-fs vectors.

Figure 4 is a schematic diagram of a vector containing an HIV segment, a functional nef gene, a funcitonal vpu gene, and a functional vpr gene.

Figure 5 is a schematic diagram of a portion of the HXB-BH8 vector.

Figure 6 is a chart of p24 production in peripheral blood lymphocytes (PBL) over time by a variety of vectors.

Figure 7 is a chart of p24 production in monocyte/macrophage cultures transfected by various vectors over time.

Figure 8 is a chart of p24 production in PBL transfected by various vectors over time.

Detailed Description of the Invention

We have now produced a vector containing a sufficient number of nucleotides corresponding to an HIV genome to express HIV gene products necessary for viral replication and infectivity (referred to as the HIV segment) and a sequence of nucleotides corresponding to a nef gene of the ELI strain to enhance viral replication (referred to as the ELI nef gene segment).

Preferably, the HIV segment corresponds to nucleotides of the HIV-1 or HIV-2 genomes. More preferably, the HIV segment corresponds to nucleotides of the HIV-1 genome. Preferably, the HIV segment does not correspond to the entire ELI strain of HIV-1.

The term corresponding means that conservative additions, deletions and substitutions are permitted.

The nef gene of ELI has its initiation codon located at position 8822, which is immediately after the end of the env

reading frame and extends through most of the U3 region of the 3' LTR. This gene encodes a protein with an apparent molecular weight of about 25-27 kd.

Most studies of nef function have used clones derived either from the prototype American IIIB strain (Gallo, R.C., et al. Science 224:500 (1984); Ratner, L., et al., Nature 313:277 (1985)], the French strain LAV BRU [Barre-Sinoussi, F., et al., Science 220:868 (1983); Wain-Hobson, S., et al., Cell 40:19 (1985)], which shares approximately 98 per cent sequence homology with IIIB, or the American SF-2 strain [Levy, J.A., et al., Science 229:840 (1984)]; Sanchez-Pescador, R., et al., Science 227:484 (1985)], which is over 90 percent homologous with both IIIB and BRU at the DNA level. All of these are strongly T-cell tropic strains. In contrast, LAV ELI is one of the more divergent clones in contrast to the IIIB, SF-2 and BRU strains. The clone is intact for all the known HIV regulatory functions [Cohen, E., et al., Nature 334:532 (1988); Cohen, E.A., et al., J. AIDS, 3:11 (1990), Alizon, M., et al., Cell, 46:83 (1986) which are incorporated herein by reference]. LAV ELI a French clone from a Zairian isolate was isolated in 1983 from a 24 year old woman with AIDS by co-cultivation of patient peripheral blood lymphocytes with fresh PHA-stimulated PBMC. There are 47 differences in amino acid sequence (out of a total of 207) between the nef product of LAV ELI and that of the IIIB-derived Hxorf clone used in our previous studies of nef [Terwilliger, E.F., et al., J. Virol. 60:754 (1986)].

In preparing a vector according to the present invention containing a functional nef segment many techniques are possible. For example, one can use a clone, e.g., LAV ELI, which contains a functional nef gene. One can obtain this

segment from the LAV ELI proviral clone by standard techniques such as by use of restriction endonucleases. For example, there is a native BamHI site at position 8506 and a SacI site at position 9607. This segment will contain the entire nef gene. One can trim this segment so that it corresponds only to the ELI nef gene segment and not to most of the other ELI gene segments by standard techniques. For example, one can insert an Eco RI site position 8792 which is only 30 base pairs upstream from the nef initiation codon. One can also use one of the other HIV clones containing nef sequences more homologous to the ELI strain than the IIIB strain. As used herein, these nucleotide sequences based upon nef sequences more homologous to the ELI strain than the IIIB strain, enhance viral replication and are considered equivalent to ELI nef. This property can readily be determined by the skilled artisan based upon the present disclosure. Alternatively, one can chemically synthesize a functional "ELI nef segment" nucleotide segment. Other techniques known to the skilled artisan based on the present disclosure can also be used to prepare the ELI nef segment.

This ELI nef segment can preferably be inserted in the presently described vector containing the HIV segment in the location where the nef segment from the other HIV-1 or HIV-2 strains used would be. If one used an HIV segment with a nef gene, one can excise that nef gene and insert the ELI nef segment.

The HIV segment will contain a sufficient number of nucleotides corresponding to all HIV genes necessary for replication and infection. Preferably this segment will also contain nucleotides corresponding to a functional vpu gene or a functional vpr gene. More preferably, it will contain

nucleotides corresponding to both functional vpu and vpr genes.

For example, the vpu sequence in the HXB2 provirus does not contain an initiation codon for vpu and therefore does not express a functional vpu gene. When the HIV segment has nucleotides corresponding to HXB2 strain one would insert an initiation codon immediately upstream and in frame with the vpr sequence. Alternatively, one could substitute a sequence of DNA containing a functional vpu gene for a sequence that does not contain a functional vpu gene. This can be done by taking advantage of various restriction sites in the nucleotide sequence. The clones BH8, BH10, LAV ELI, etc. contain a nucleotide sequence which results in a functional vpu gene.

The HXB2 strain has a premature termination codon and does not express a functional vpr protein. If one is using an HIV segment that corresponds to such a vpr gene, which is not considered functional, one can insert a functional vpu gene from a strain that expresses a functional vpr gene product such as LAV BRU, LAV ELI, etc.

As aforesaid, vectors can be synthesized by a variety of methods known to the person of ordinary skill in the art. A variety of different vectors which can be prepared are shown in Figures 1-4.

Different combinations of sequences containing the ELI nef sequences can be constructed and inserted within the 3' end of an HIV sequence, such as one having nucleotides corresponding to the replication competent HXB2 (also referred to as HXBc2) provirus. Compatible restriction sites

can be generated by standard techniques where needed such as by oligonucleotide-directed mutagenesis. For example, a BamHI site within LAV ELI and a new Eco RI site within both the LAV ELI and HXB2 sequences. One preferably takes care to make sure that with each nucleotide change made for "inserting" a restriction site, the change does not alter the predicted amino acid sequences encoded by any gene or at a minimum results in a conservative amino acid change. Constructs can be confirmed by sequencing. HXB-ELI 1 consists entirely of HXB2 sequences as far 3' as the native BamHI site at position 8506, within the env and rev gene sequences, upstream of nef, and LAV ELI sequences between that position and a Sacl site at position 9607. The remainder of the 3' LTR sequence is derived from HXB2. See Figures 1 and 2. In HXB-ELI-2 the 5' junction between the HXB2 and LAV ELI sequences is shifted downstream to a new Eco RI site at position 8792, only 30 base pairs (bp) upstream from the nef initiation codon. See Figures 1 and 3. HXB-ELI-2 contains only HXB2 sequences as far 3' as this Eco RI site. The LAV ELI sequence extends from this position 3' to the Sac 1 site at 9607, as in the case of HXB ELI-1. These vectors do not contain functional vpr or vpu genes. However, functional genes can readily be added as discussed above. For example, excising the sequence in these vectors corresponding to the sequence between an Apa 1 site at position 2008 and a Sal 1 site at position 5820 and replacing it with the corresponding sequence from clone LAV BRU, which includes a functional vpr reading frame.

Similarly, the sequence from the vector corresponding to a sequence between the Sal 1 site 5820 and a kpn 1 site at position 6382 can be excised and replaced with the corresponding segment from BH10, which includes a functional vpu gene. As discussed above, other strategies and

modifications of this strategy can be used by the skilled artisan based upon this disclosure.

Proviral plasmids containing the ELI nef gene and the HIV segment vector described above can readily be made by inserting this sequence into a plasmid backbone by known means such as use of restriction enzyme sites.

As used herein the term a sufficient number of nucleotides permits additions, deletions and substitutions as long as the claimed functional ability is not lost. For example, if one is referring to the functional ability of the nef gene in terms of viral replication, then replication of the resultant virus must be greater than by an isogenic nef virus.

The vector is used to transfect a desired cell by standard techniques. For example, one can transfect a cell in vitro using, for example, the calcium phosphate coprecipitation technique or DEAE dextran technique. Alternatively, this vector can be used to transfect living cells in vivo. Such techniques are well known to the skilled artisan.

By use of the nef gene segment of the present invention, one is able to take an HIV strain, for example, the HXB2 strain, which does not replicate well in peripheral blood mononuclear cells (PBMC) as well as purified primary human monocytese or monocytoid cell lines and obtain enhanced infectivity in such cell lines.

Although not wishing to be bound by theory, it appears that there is a complex series of interactions between the

various genes in the HIV genome, which may be responsible for some of the more acute effects seen with the virus. For example, one of the strategems proposed by which HIV evades the immune response in infected individuals is by establishment of a low-level infectious state in which the virus either lies dormant or replicates at a very low level within most infected cells. Accordingly, the virus can establish a stable infection via integration of the virus into the host DNA. In order to accomplish such an objective, the native virus is initially carefully regulated so that it does not appear too "aggressive". By taking the nef gene segment of the present invention and inserting it with other strains or an HIV-2 genome, some of the interactions are believed to be overcome. Thus, the provirus formed by the vectors will be more aggressive. For example, we believe that the IIIB precursor of HXB2 spontaneously mutated to a nef$^-$ phenotype. This nef$^-$ precursor of HXB2 replicated poorly in PBMC. A compensatory mutation arose in 3' env sequences of the precursor to HXB2 to produce a virus that could replicate well in PBMC. The HXB2 virus contains the original nef mutation as well as the compensatory mutation in 3' env sequences. Furthermore, the 3' env compensatory mutation has a negative affect on growth in the presence of closely related nef sequences such as the BH8 and BH10 nef genes. This negative interaction does not extend to divergent nef sequences such as the ELI nef gene.

Consequently, sequences in HIV outside of nef affect nef function. There are differences in nef alleles with respect to interaction with the extragenic sequences. BH8 and BH10 nef is a negative regulator with HXB2 3' env sequences. BH8 and BH10 nef should be positive regulators with ELI 3' env sequences. ELI nef is a positive regulator with either HXB2

or ELI 3' env sequences. ELI nef is an essential gene for growth in primary monocytes and macrophages. Accordingly, various tropisms exhibited by HIV strains will not be exhibited by vectors of the present invention. Thus, such vectors can be used to transform a wider range of cells than the strains typically can.

One can use the present vector to generate an antigenic response in an animal by standard techniques and then use the antibody generated in treatment.

It has been reported that a cloned SIV_{mac} virus containing a simple frameshift mutation in nef has a strong tendency to back-revert in vivo, with restoration of the nef reading frame. If, as is often proposed, monocytes are a primary initial target of HIV infection in vivo and/or infected macrophages form an important long term reservoir for maintaining a large pool of virus within the system, our discovey explain why a strong selection pressure for an intact nef function would exist in vivo, but not necessarily in vitro, namely the tropism shown by the ELI nef gene.

Although not wishing to be bound by theory, we believe that major variations in tropism for different types of CD4-positive cells is in fact a distinct virus-regulated phenomenon.

Using this vector to transfect cells in vitro or in vivo can permit an improved system for screening drugs and understanding the pathogenesis of the virus. Since the range of the cells transformed by this vector is wider than that with other laboratory strains such as the IIIB strain, one is able to explore the affects of a drug on the spread of the

virus in a wider range of infected cells than is typical.

For example, it can be used in studies in primary cell cultures to determine the mechanism by which the ELI nef allows high-level replication in monocytes. Such information will be of vital relevance to understanding the mechanisms of disease progression in AIDS, particularly in light of evidence suggesting that monocytropic strains of the virus may play a key role in development of the central nervous system disorders often accompanying HIV infection, independent of whether immunological impairment is evident. [Koyanagi, Y., et al., Science 236:819 (1987)].

In one assay one can add a predetermined compound to a cell or bodily fluid and thereafter attempt to transfect those cells with a vector of the present invention. One would add an amount of the vector to the cell that had previously been determined to be effective in transforming such a cell. Thereafter one would measure the level of infectivity of that cell to determine the effectiveness of a compound in preventing the spread of the virus. Alternatively, one can first transfect the cell either in vitro or in vivo with a vector of the present invention thereafter add the predetermined compound to the cells and then determine the effectiveness of that compound in preventing the spread of the virus. The parameters observed include syncytia formation, single-cell killing, levels of supernatant of HIV p24 core protein such as determined by RIA, immunoprecipitation of metabolically labelled viral proteins with AIDS patient serum, etc.

The vector is preferably used in vitro with primary monocytes/macrophages.

The vector can also be used in vivo to "infect" cells. In general, the traditional laboratory strains of HIV-1 have replicated very poorly in traditional animal model systems, for example, in the SCID-HU mouse system which several investigators and companies are attempting to develop into a viable animal model for certain aspects of HIV pathogenesis. As aforesaid, the present vectors result in proviral clones which replicate much more aggressively in primary lymphocytes than the typical lab strains and should replicate much more effectively in the SCID-HU mice. Other preferred animal models include mammals other than humans. Preferred examples of such mammals would include rabbits, mice, rats, cats and primates. Preferred primates include chimpanzees and monkeys.

One can readily determine an effective amount of vector necessary to administer the animal to establish infection. For example, 0.1 ug to about 10 mg of vector per kg body weight. Administration can be any of a variety of routes including parenteral administration such as intramuscular, intraperitoneal, intravenous or subcutaneous.

Knowing the amount and the conditions necessary to establish "infection" (transfection) permits the development of a model assay to establish effectiveness of a preselected compound in vivo. One would adminster the compound to the animal by standard means, such as injection. Thereafter, one would administer a sufficient amount of the vector to normally establish an infection and observe the animal to determine whether an infection develops, and if it does, the degree of infection. Alternatively, one can first administer a sufficient amount of the vector to establish an infection in the animal and then administer the preselected compound.

Thereafter, one would observe the animal to determine the effect of the preselected compound on the "infection" by the vectors.

In another embodiment, one can prepare a vector comprising a sufficient number of nucleotides corresponding to a nef gene of the ELI strain of HIV-1 to enhance viral replication (ELI nef gene segment), a promoter sequence and a polyadenylation sequence, but not containing all the ELI encoding nucleotide sequences. Preferably, the vector contains an LTR sequence, more preferably an HIV LTR sequence. The promoter can be the LTR or a separate promoter such as another viral promoter, e.g., the SL3 promoter. This vector would preferably contain a heterologous gene. In one embodiment, the heterologous gene would be a marker gene. Marker genes are well known in the art, for example, the chloramphenicol acetyltransferase (CAT) gene or a growth hormone such as human growth hormone (hGH). The vector can be cotransfected into a cell with a vector comprising a replication competent HIV provirus, or alternatively into a cell line transfected by a replication competent HIV, e.g., HXB2 or HXB-BH8. This would permit further anlaysis of the interaction between the ELI nef segment and other viral genes. The use of this vector should also increase the range of host that can be transfected by such virus.

The present invention is further illustrated by the following examples. These examples are provided as an aid to understanding the invention, and are not to be construed as a limited thereof.

Examples

Construction of Vectors

HXB2 is a full-length infectious provirus derived from the IIIB strain of HIV-1 [Fisher, A.G., et al., Nature 316:262-265 (1985)] its nef, vpr and vpu genes are defective as a consequence of single point mutations in each gene. There is a BamHI site at nucleotide 8506, whose location was used in preparing a variety of plasmids. See Figure 1.

Compatible restriction sites were generated where needed by oligonucleotide directed mutagenesis - specifically a BamHI site within LAV ELI and a new Eco RI site within both the LAV ELI and HXB2 sequences. In each instance changes were made which did not alter the predicted amino acid sequence encoded by any gene. All constructs were confirmed by sequencing.

HXB-ELI 1 consists entirely of HXB2 sequences as far 3' as the native BamHI site at position 8506, within the env and rev gene sequences upstream of nef, and LAV ELI sequences between that position and a SacI site at position 9607. See Figures 1 and 2. The remainder of the 3' LTR sequence is derived from HXB2.

In HXB-ELI-2 the 5' junction between the HXB2 and LAV ELI sequence is shifted downstream to a new Eco RI site at position 8792, only 30 bp upstream from the nef initiation codon. See Figures 1 and 3. HXB-ELI-2 contains only HXB2 sequences as far 3' as this Eco RI site. The LAV ELI sequence extends from this position 3' to the SacI site at 9607, as in the case of HXB-ELI-1.

The substitution by the ELI sequences also introduced

changes within the second coding exon of rev [Sodroski, J.G., et al. Nature 371:412 (1986)] and that portion of the env gene encoding the carboxy terminus of the transmembrane protein, as well as the U3 region of the 3' LTR which overlaps nef. A frameshift was therefore introduced within the nef reading frame of the recombinant to generate a second isogenic clone containing a dysfunctional nef-HXB-ELI 1-nef. Four base pair frameshifts were introduced within the nef coding sequences of HXB-ELI-1 and 2 by filing out the native XhoI site at position 8928 to generate isogenic nef-defective proviruses. See Figures 2 and 3, respectively.

In HXB-BH8, the 3' terminus at the native XhoI site contained sequences derived from the BH8 provirus. This strain is a IIIB strain, which contains a complete nef open reading frame and was originally referred to as HXB-orf. See Figure 5. The vpr^+, vpu^+ HXB-BH8-R provirus was derived from the vpr^-, vpu^- HXB-BH8. The proviral sequence of HXB-BH8 between an Apa 1 site at position 2008 and a Sal 1 site at position 5820 was replaced with the corresponding sequence from clone LAV BRU. This segment includes a functional vpr reading frame. The HXB-BH8 sequence between 5820 and a kpn 1 site at position 6382 was then excised and replaced with the corresponding segment from BH10, a closely related IIIB clone. This segment includes a functional vpu gene. The same replacements were made in HXB-ELI 1 to generate HXB-ELI 1-R. See Figure 4.

The HXB-ELI 1-R sequence was inserted into the known plasmid pBR322 between the EcoRV and PvuII sites to generate the plasmid DFCI-HT, which contains the HXB-ELI-1-R sequence with a pBR322 backbone.

A sample of this plasmid has been deposited with the American Type Culture Collection (ATCC) under the Budapest Treaty and given ATCC Accession No. 68343.

Transfection by Vectors

10^7 Jurkat cells were tranfected by the DEAE-dextran procedure on Day 0 with 10 mg of each proviral plasmid as described by Lopata, et al., Nucl. Acids Res. 12:5707-5717 (1984); Queen and Baltimore, Cell 33:741-748 (1983); Sodroski, J., et al., Science 231:1549-1553 (1986). Cultures were maintained thereafter in 12.5 ml of RPMI +15% fetal calf serum, and given a complete medium change daily. Aliquots of each supernatant were saved at the time of each feeding for later assay. Cultures were monitored for syncytia formation and total cell number in addition to levels of HIV p24 core protein the supernatant.

Aliquots of each transfected Jurkat culture were metabolically labelled, and collected for immunoprecipitation as described in Terwilliger, et al., J. Virol. 60:754-760 (1986). One-half of each sample was immunoprecipitated with AIDS patient serum. The other half was reacted with nef protein antiserum. Immunoprecipitation and polyacrylamide gel electrophoresis were performed as described by Laemmli, U.K., Nature 227:680-685 (1970).

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll separation from buffy coats obtained from healthy seronegative blood donors. The PBMC cultures were seeded at a density of 4×10^6 in 24 mm tissue culture wells (Costar, MA) in RPMI-1640 with 10% fetal calf serum and antibiotics and 20% PHA conditioned culture medium. After 48 hours of culture virus was added to each well. Titered viral stocks

were obtained from transfection of Jurkat cell cultures. Infectious supernatant equivalent to 20 ng of p24 was added to each well of primary cells. The infected cell cultures were incubated overnight and the growth medium was then carefully decanted from each well and replaced with fresh medium. After the last medium change samples for p24 assays were collected every third day before the cultures were fed. The samples were inactivated in 0.5% Triton-X and stored until assayed. p24 was measured using a commercially available RIΔkit (Dupont, NEN).

Equivalent amounts of cell-free virus were then used to infect fresh activated peripheral blood mononuclear cells (PBMC) as well as purified primary human monocytes by the same means as described above. Replication of each virus was assayed over time by measurement of p24 core antigen in the supernatants.

Monocytes were obtained from the PBMC by fractionation on Percoll gradients. Contaminating T cells were from the low density cells removed by rosetting with neuraminidase treated sheep red blood cells.

The purity of the monocyte population was verified by immunoperoxidase staining of cytopin spin cell preparations of the isolated monocytes. More than 95% of the cells stained with Leu3-M and <5% were positive for Leu 18 (CD20), Leu 3 (CD4), or Leu 2 (CD8). 4×10^6 monocytes were cultured as described for PBMC cultures to maintain a comparable growth environment. Viral stocks were used as described for PBMC cultures and samples for p24 assays were harvested at the same points.

The insertion of the 1.1 kb segment of the 3' LAV ELI sequence in creating HXB-ELI 1, as aforesaid, introduced changes within the second coding exon of rev and that portion of the env gene encoding the carboxy terminus of the transmembrane protein, as well as the U3 region of the 3' LTR which overlaps nef. The frameshift vector HXB-ELI 1-fs was generated to result in an isogenic clone containing a dysfunctional nef. This pair of provirus constructs was then transfected into Jurkat cells as described above and the kinetics of virus replication were compared.

The pair of IIIB-derived nef-positive (HXB-BH8) and negative (HXB2) viruses were also transfected as controls. As discussed, HXB2 contains a point mutation within nef resulting in a premature termination codon being inserted into the reading frame after amino acid 123. This truncated nef protein is not functional. HXB-BH8 (previously called Hx-orf) does not contain this mutation, and produces a full-length nef product [Terwilliger, E.F., et al., J. Virol. 60:754 (1986)].

A lag in levels of supernatant p24 was detectable in the culture transfected with HXB-BH8 as compared to the one containing HXB2 at early times post-transfection. A marked difference in the pattern of syncytia formation was also apparent. Replication of virus derived from HXB-ELI 1 following transfection was not substantially different from that of HXB2. In different experiments the kinetics of virus infection exhibited by HXB-ELI 1 were either equivalent to or modestly enhanced over those displayed by HXB2.

However, by every parameter measured, the replication of virus derived from the isogenic nef-defective recombinant

provirus (HXB-ELI 1-fs) was markedly attenuated. Supernatant p24 levels in the nef-negative virus culture were depressed up 100 fold in this experiment compared with the isogenic nef-positive virus culture. Peak cell-associated viral protein production was also dramatically decreased in this virus culture, indicated by the fainter viral bands in the immunoprecipitation profiles. These measurements paralleled a dramatic attentuation of the cytophathic effect of the HXB-ELI-nef$^-$ virus upon the infected cells, whether evaluated by syncytia formation or by decreases in total cell numbers over time. Similar results were obtained in repeat experiments, sometimes with even larger differences in kinetics between the nef-positive and nef-negative HXB-ELI 1 clones. Immunoprecipitation of metabolically labelled infected cell lysates with a nef-specific polyclonal rabbit serum revealed the presence of a 27kD nef protein in lysates from cells infected with either the HXB-BH8 or HXB-ELI 1 viruses. This band was not visible in lysates from cells transfected with either HXB2 or HXB-ELI 1-fs.

Transfection by HXB-BH8 confirmed the earlier report that introduction of a functional nef gene into the HXB2 provirus results in a virus which replicates more slowly on CD4$^+$ T cells than does the nef$^-$ counterpart.

The LAV ELI nef gene appeared to display a marked enhancing effect upon the replication of the virus in the Jurkat cells. This positive effect was larger and in the opposite direction from the smaller negative effect of IIIB nef observed in the context of the HXB-BH8/HXB2 pair of viruses. A repeat experiment using independently derived clones of the HXB-ELI 1 pair of proviruses yielded comparable results, eliminating the possibility that a random secondary

change at some distal site within one of the constructs was responsible for the results.

To minimize the potential for generating artifacts in our experimental design, additional provirus constructs were prepared as described above. The kinetic studies were also extended into freshly cultured primary cells challenged with cell-free virus, as opposed to transfected stable T-cell lines. HXB-ELI 2 contains a smaller insertion of LAV ELI sequences than HXB-ELI 1 (See Figure 1 and 3). The 3' end of the 800 base pair insert remains the same but the 5' end is shifted downstream to just 30 base pairs 5' to the nef initiation codon. In addition to the 47 differences in the predicted amino acid sequences of the nef proteins between HXB-ELI 2 and HXB2, there is only a single amino acid change in the predicted env product of HXB-ELI 2. An isoleucine in the HXB2 env product at position 854, only 2 residues before the carboxy terminus of gp41, is converted to a serine in HXB-ELI 2. There are no changes in any other coding frames. An isogenic nef-defective form of the IIXB-ELI 2 provirus was also generated by insertion of a frameshift into nef, HXB-ELI 2-fs as described above.

As aforesaid, HXB2 is defective in two other regulatory genes in addition to nef, the vpu [Cohen, E.A., et al., J. AIDS 3:11 (1990)] and vpr [Alizon, M., et al., Cell 46:83 (1986)] genes. In the HXB-BH8-R (for restored) provirus, which was derived from HXB-BH8, intact vpr and vpu frames have been restored by recombination with sequences from other IIIB clones and LAV ELI as described earlier (See Figure 1). HXB-BH8-R therefore represents a provirus which would be predicted to be fully functional for all the HIV genes. Identical recombinations were made in HXB-ELI 1 to generate

HXB-ELI 1-R. See Figure 4. HXB-BH8-R and HXB-ELI 1-R are therefore isogenic except for the 1.1 kb of LAV ELI sequence in HXB-ELI 1-R which replaces the corresponding IIIB sequence in HXB-BH10-R.

IIIB replicates very well in T-cell lines but poorly in PBMC, and is particularly weak in either primary monocytes or monocytoid cell lines [Koyanagi, Y. et al., Science, 236:819 (1987)]. Barely detectable levels of p24 antigen production were observed within the supernatants of PBMC infected with the HXB2 virus. See Figure 6. Similarly, the HXB-BH8 and HXB-BH8-R viruses also did not exhibit significantly increased activity in the PBMC over HXB2 (Figure 6). No detectable replication by any of these three viruses was observed in the monocyte macrophage cultures (Figure 7).

Figures 6 and 7 both show p24 production over time for cells transfected with HXB-ELI 1 (ELI-1 nef^+, open circle), HXB-ELI 2 (ELI-2 nef^+; solid square) HXB-BH8 (BH8 nef^+, open square); HXB-ELI 1-fs (ELI-1fs nef^-, solid circle); HXB-ELI 2-fs (ELI-2fs nef^-, solid triangle) and HXB2 (HXBc2 nef^-, open triangle).

In contrast, HXB-ELI 1 and HXB-ELI 2 viruses exhibited strong replication within the PBMC, attaining maximum levels of p24 antigen production, of ca. 26 ng/ml and ca. 33 ng/ml respectively, on Day 17 and 20 following infection (Figure 6). In repeat experiments there was no consistent difference in the level of activity of the two viruses. The HXB-ELI 1-R virus was even more aggressive, achieving a peak virus production level 3-5-fold higher than HXB-ELI 1 or ELI 2. This was more than 50-fold higher than the maximum virus titer attained by any of the viruses containing IIIB nef

sequences. In contrast, in cultures infected with either of the isogenic nef-frameshift HXB-ELI 1-fs and HXB-ELI 2-fs viruses, supernatant p24 levels were again either barely within or below the limit of detection.

All three of the nef-positive HXB-ELI recombinant viruses replicated with great vigor within the primary monocyte cultures. For example, the HXB-ELI 1 in a typical experiment reached peak production levels of about 35 ng/ml in the monocytes on Day 20 following infection. HXB-ELI 1-R again attained a several-fold higher maximum titer than the other two viruses. No p24 could be detected in the supernatants of monocytes infected with either of the HXB-ELI-nef-defective isogenic viruses. See, for example, Figure 8, where HXB-ELI 1-R is represented by open squares, HXB-ELI by open triangles and HXB2 by open circles, which shows the results of another typical experiment.

The results from the Jurkat, PBMC, and monocyte infections all highlight a dramatic change in the behavior of the LAV ELI recombinant viruses as compared with those viruses containing no LAV ELI sequences, and specifically indicate that this change requires the presence of the intact LAV ELI nef gene. Viruses such as HXB-BH8 in which the nef coding sequences were intact but contributed by IIIB did not exhibit this new phenotype.

This difference is evident even when comparing the isogenic nef-positive and nef-negative viruses in the Jurkat cells, where IIIB nef is seen to exert a small negative effect upon virus replication while LAV ELI nef produces a larger positive effect. Most striking however, the presence of the intact LAV ELI nef gene clearly confers upon the

recombinant viruses a new tropism for monocytic cells, which is also reflected in their capacity for vigorous replication in the PBMC. The level of virus production in infected monocytes by the HXB-ELI 1-R virus is as high as any reported for the supposedly monocytropic HIV strains [Koyanagi, Y., et al., Science, 236:819 (1987), Schwartz, S., et al., PNAS, 86:7200 (1989), Collman, R., et al., J. Exp. Med. 170:571 (1989)].

Although possible that additional overlapping elements for example, within the U3 region of the 3' LTR may contribute to the changed phenotype of the LAV ELI recombinants, because HXB-ELI 2 appears to replicate as well in the monocytes as HXB-ELI 1, the additional sequence differences between HXB2 and HXB-ELI 1 present in the env and rev genes do not appear to contribute to the phenomenon. It is clear, however, from the negative results obtained with both of the nef-defective LAV ELI viruses that the intact LAV ELI nef is essential for the phenotype. In contrast, while there was an approximately 5-fold enhancement in the level of virus production in the primary cells by the vpu-positive, vpr-positive HXB-ELI 1-R virus compared to the parental HXB ELI 1, which is defective for both these functions, restoration of vpu and vpr in HXB-BH8 did nothing either to alleviate the block to replication by this virus in the monocytes nor to boost the low level of infection in the PBMC. As previously reported, each of these factors functions to enhance virus replication [Cohen, E.A., et al., J. AIDS, 3:11 (1990), Alizon, M., et al., Cell, 46:83 (1986)]. While coding changes in other reading frames have also been introduced into HXB-ELI 1-R as compared to the original HXB-ELI 1, the magnitude of the effects we have characterized for the vpu and vpr during virus infection of

Jurkat cells is entirely sufficient to account for the boost in virus titers seen in both the PBMC and monocyte cultures infected with HXB-ELI 1-R.

We believe that major variations in tropism for different types of CD4-positive cells in in fact a distinct naturally occurring, virus-regulated phenomenon.

We successfully duplicated the observation of other investigators that nef can modestly down regulate expression from the HIV LTR in trans. This was accomplished by co-transfection into COS-7 cells of an HIV LTR-CAT construct together with either a nef expression vector or an isogenic vector containing a frameshift in the nef seqeunce. Each plasmid included an SV40 origin of replication. However, similar results were obtained using HIV LTR elements cloned from either HXB2 or LAV ELI, and regardless of which strain of nef was being expressed. Together with the replication studies, this suggests that the down regulatory effects may be secondary phenomena.

It is evident that those skilled in the art, given the benefit of the foregoing disclosure, may make numerous modifications thereof and departures from the specific embodiments described herein, without departing from the inventive concepts and the present invention is to be limited solely by the scope and spirit of the appended claims.

We claim:

1. A vector comprising:

(a) a sufficient number of nucleotides corresponding to an HIV genome to express HIV gene products necessary for viral replication and infectivity, the HIV segment, but not corresponding to all the genes necessary for viral replication and infectivity from the ELI strain; and

(b) a sufficient number of nucleotides corresponding to a nef gene of the ELI strain of HIV-1 to enhance viral replication, the ELI nef gene segment.

2. The vector of claim 1, wherein the HIV segment contains a sufficient number of nucleotides corresponding to a functional HIV vpu gene to produce functional vpu protein.

3. The vector of claim 1, wherein the HIV segment contains a sufficient number of nucleotides corresponding to a functional vpr gene to produce functional vpr protein.

4. The vector of claim 3, wherein the HIV segment contains a sufficient number of nucleotides corresponding to a functional vpu gene to produce functional vpu protein.

5. The vector of claim 1, wherein the HIV segment corresponds to a sufficient number of nucleotides to produce functional env protein, but wherein it does not correspond to the env nucleotides of the ELI strain.

6. The vector of claim 1, wherein the HIV segment corresponds to a sufficient number of nucleotides to produce functional gag protein, but wherein it does not correspond to the gag nucleotides of the ELI strain.

7. The vector of claim 1, wherein the HIV segment corresponds to a sufficient number of nucleotides to produce functional pol protein, but wherein it does not correspond to the pol nucleotides of the ELI strain.

8. The vector of claim 1, wherein the HIV genome is the HIV-2 genome.

9. The vector of claim 1, wherein the HIV segment corresponds to nucleotides of the HIV-1 genome.

10. The vector of claim 1, wherein the HIV segment contains a sufficient number of nucleotides corresponding to the gag gene, the pol gene, the env gene, the rev gene, the tat gene, the vpu gene, and the vpr gene to produce functional gag, pol, env, rev, tat, vpu and vpr proteins.

11. A vector comprising:

(a) a sufficient number of nucleotides corresponding to a nef gene of the ELI strain of HIV-1 to enhance viral replication, the ELI nef gene segment;

(b) a promoter sequence; and

(c) a polyadenylation sequence;

wherein the vector does not contain all the ELI encoding nucleotide sequences.

12. The vector of claim 11, wherein the vector contains a sufficient number of nucleotides corresponding to an LTR sequence.

13. The vector of claim 11, wherein the vector also contains a heterologous gene.

14. A method of assaying in vivo for compounds effective against infection by HIV virus, which comprises:

(a) administering to an animal the vector of claim 1, in a sufficient amount to infect that animal;

(b) adding a predetermined compound to the animal, and

(c) observing the animal to determine the effect of the predetermined compound on infection by the vector.

15. A method of assaying for the effectiveness of compounds against infection by HIV virus, which comprises;

(a) adding a predetermined compound to a preselected animal;

(b) administering to the preselected animal the vector of claim 1 in an amount which has been determined as sufficient to cause infection, and

(c) observing the animal to determine the effect of the preselected compound on infection by the vector.

16. The method of claim 14, wherein the animal is a mammal, other than a human.

17. The method of claim 16, wherein the mammal is a primate.

18. The method of claim 16, wherein the mammal is selected from the group consisting of rabbits, mice, rats, cats, chimpanzees, and monkeys.

19. The method of claim 14, wherein the animal is a chimpanzee.

20. The method of claim 15, wherein the preselected animal is a mammal, other than a human.

21. The method of claim 15, wherein the mammal is a primate.

22. The method of claim 15, wherein the mammal is selected from the group consisting of rabbits, mice, rats, cats, chimpanzees, and monkeys.

23. The method of claim 15, wherein the preselected animal is a chimpanzee.

24. A method of assaying for compounds effective against HIV virus, which comprises;

(a) transfecting a cell line with a sufficient quantity of the vector of claim 1 to obtain infected cultures;

(b) adding a predetermined compound to the infected cultures; and

(c) screening the cultures to determine the effect of the compound on the transfected cultures.

25. The method of claim 24, wherein the cell line is a primary monocyte.

26. A method of assaying for the effectiveness of a compound against the infectivity of HIV virus, which comprises;

(a) adding a predetermined compound to a culture of cells;

(b) attempting to transfect the culture of cells of step (a) with the vector of claim 1 in an amount, which has previously been determined to be sufficient for transfecting the culture of cells; and

(c) screening the culture to determine whether the culture has been transfected and, if it has been transfected, the extent of the transfection by the vector of step (b).

27. The method of claim 26, wherein the culture of cells is a culture of primary monocytes.

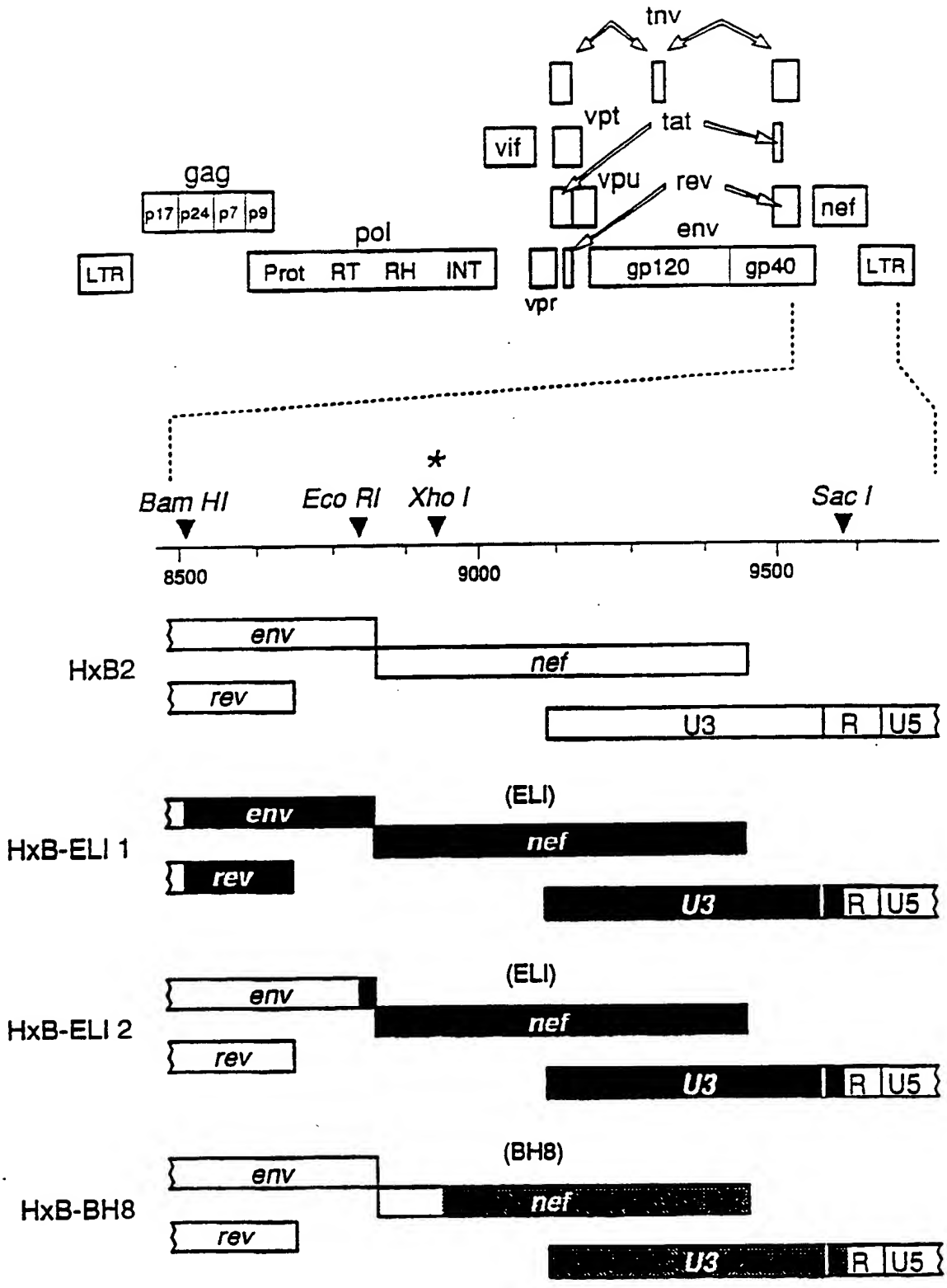

tnv
vpt
tat
vif
vpu
rev
nef
gag
p17 p24 p7 p9
pol
env
LTR
Prot RT RH INT
gp120 gp40
LTR
vpr
*
Bam HI
Eco RI
Xho I
Sac I
8500
9000
9500
HxB2
env
nef
rev
U3 R U5
(ELI)
HxB-ELI 1
env
nef
rev
U3 R U5
(ELI)
HxB-ELI 2
env
nef
rev
U3 R U5
(BH8)
HxB-BH8
env
nef
rev
U3 R U5

FIGURE 1

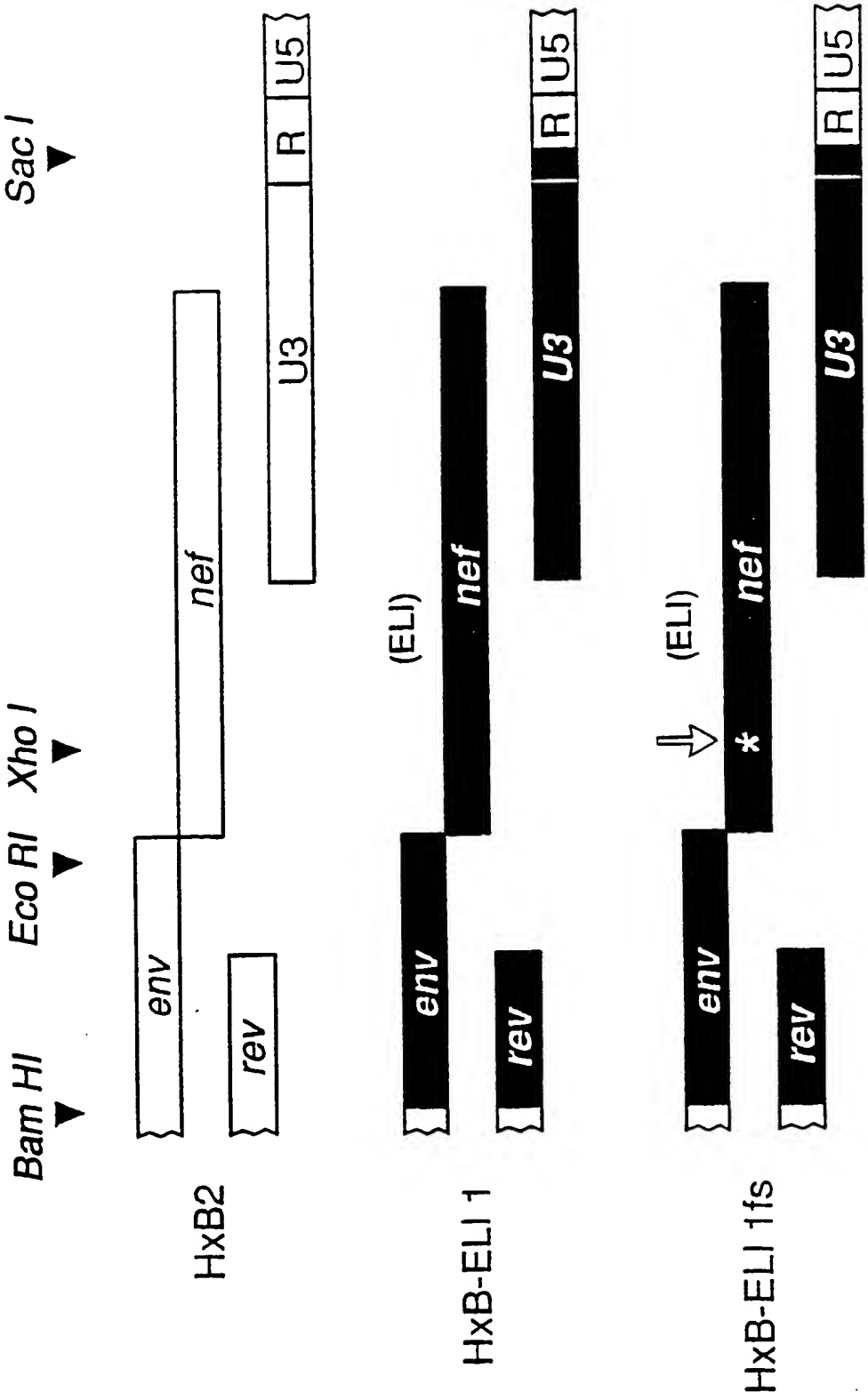

Bam HI
Eco RI
Xho I
Sac I
env
rev
nef
U3
R
U5
HxB2
(ELI)
HxB-ELI 1
HxB-ELI 1fs
*

FIGURE 2

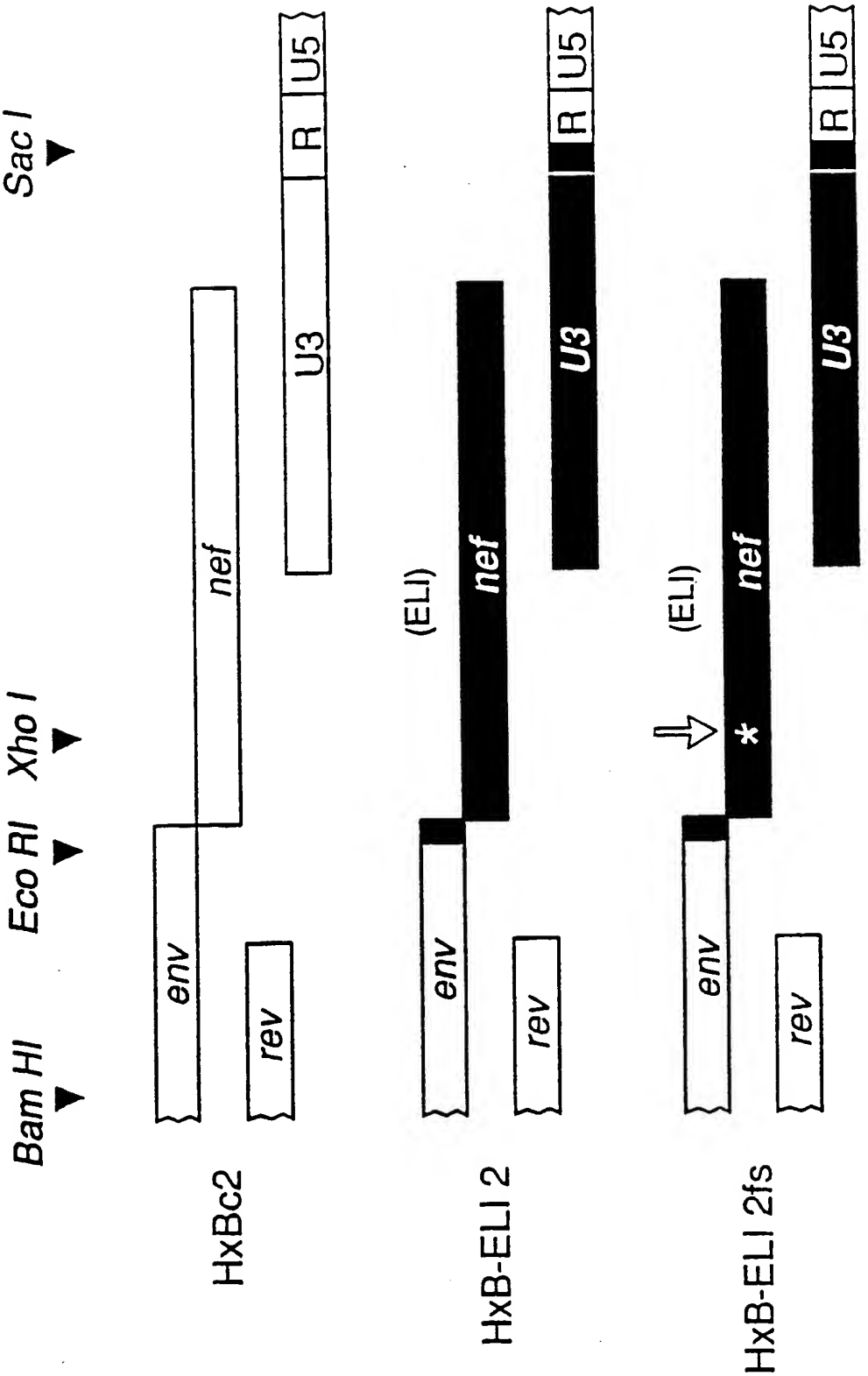
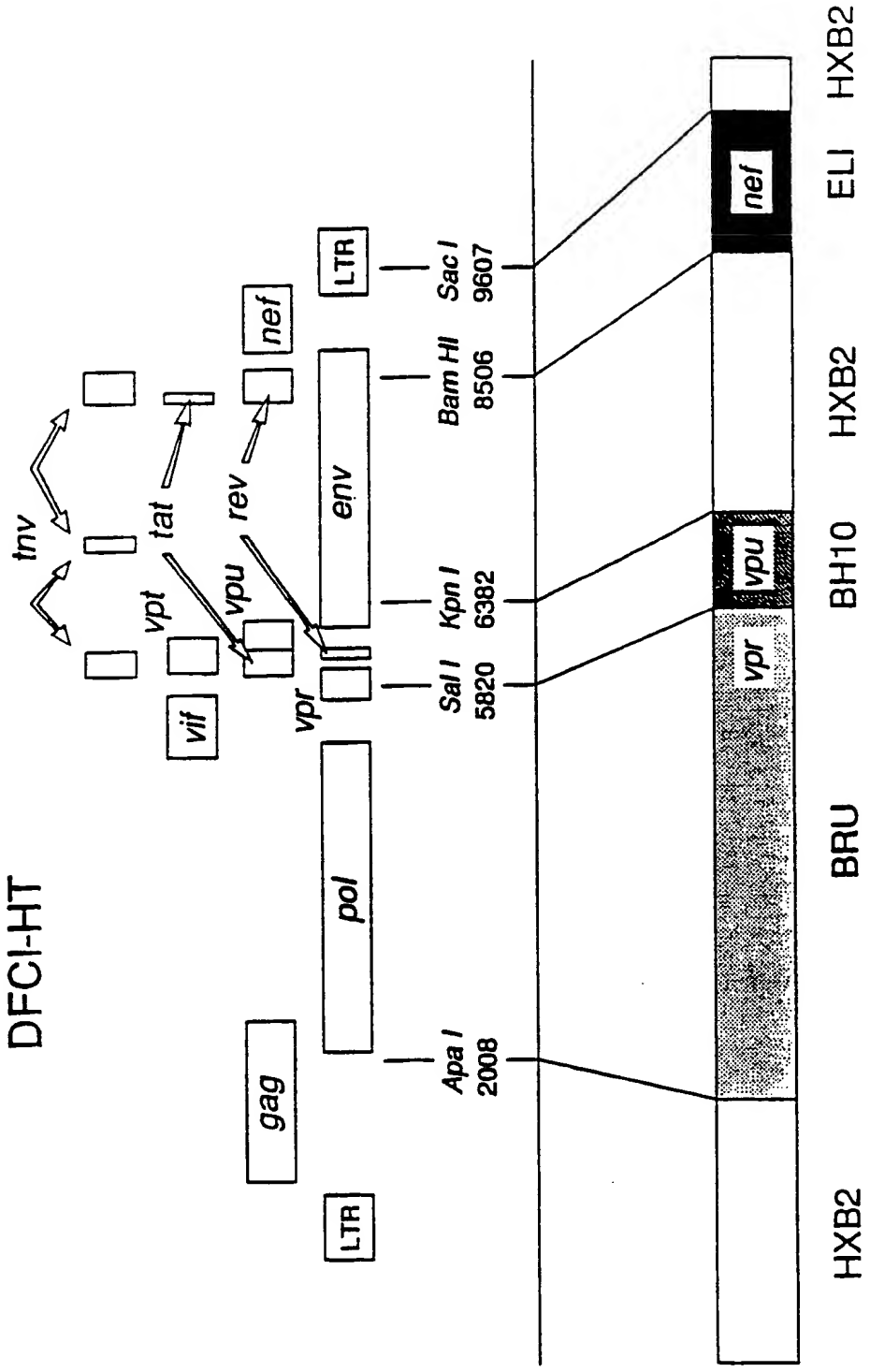

Bam HI
Eco RI
Xho I
Sac I
env
rev
nef
U3
R
U5
HxBc2
(ELI)
HxB-ELI 2
HxB-ELI 2fs
*

FIGURE 3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LTR
gag
pol
vif
vpr
vpt
tat
vpu
rev
env
nef
LTR
tnv
Apa I
2008
Sal I
5820
Kpn I
6382
Bam HI
8506
Sac I
9607
HXB2
BRU
BH10
HXB2
ELI
HXB2
vpr
vpu
nef

FIGURE 4

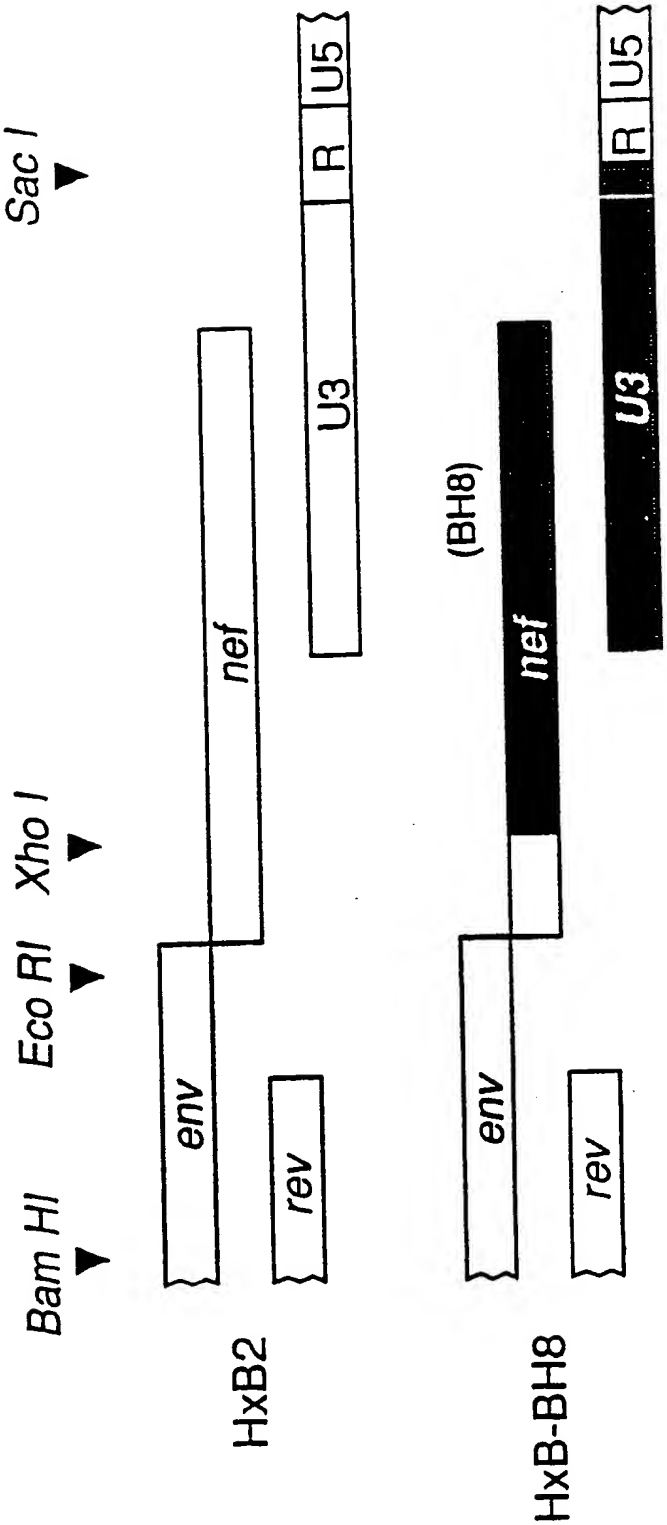

FIGURE 5

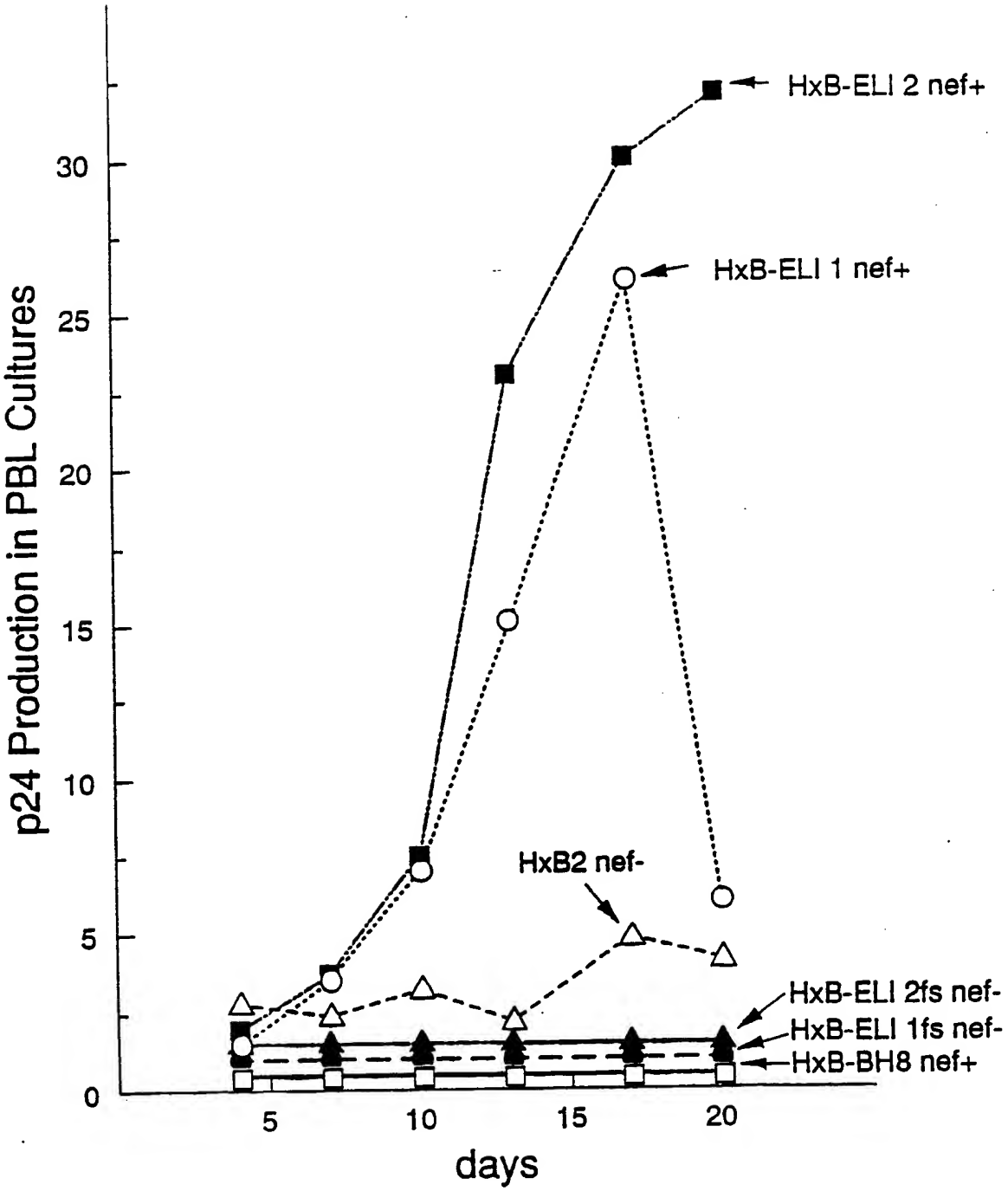
p24 Production in PBL Cultures
0
5
10
15
20
25
30
5
10
15
20
days
HxB-ELI 2 nef+
HxB-ELI 1 nef+
HxB2 nef-
HxB-ELI 2fs nef-
HxB-ELI 1fs nef-
HxB-BH8 nef+

FIGURE 6

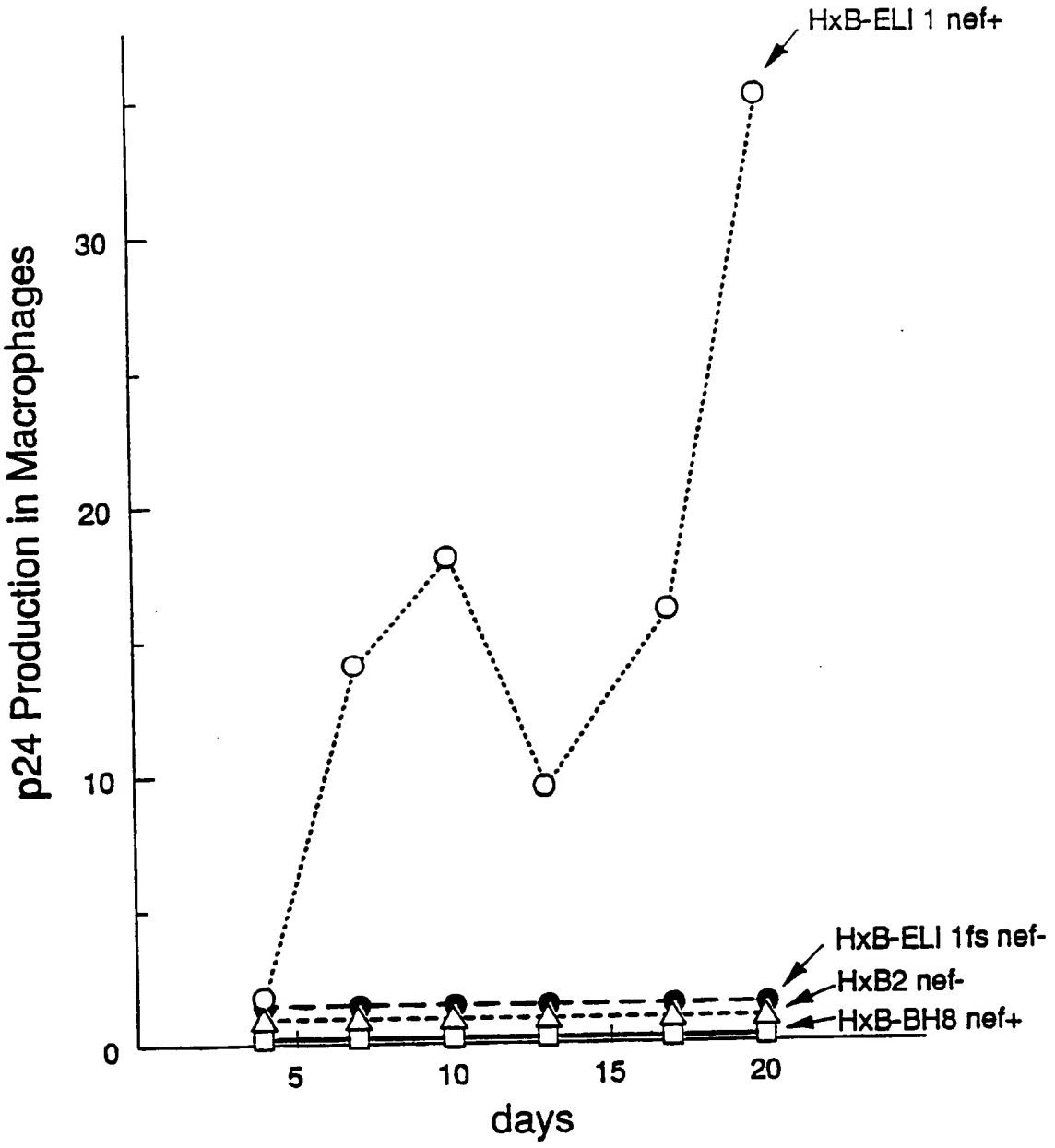
HxB-ELI 1 nef+
p24 Production in Macrophages
30
20
10
0
HxB-ELI 1fs nef-
HxB2 nef-
HxB-BH8 nef+
5
10
15
20
days

FIGURE 7

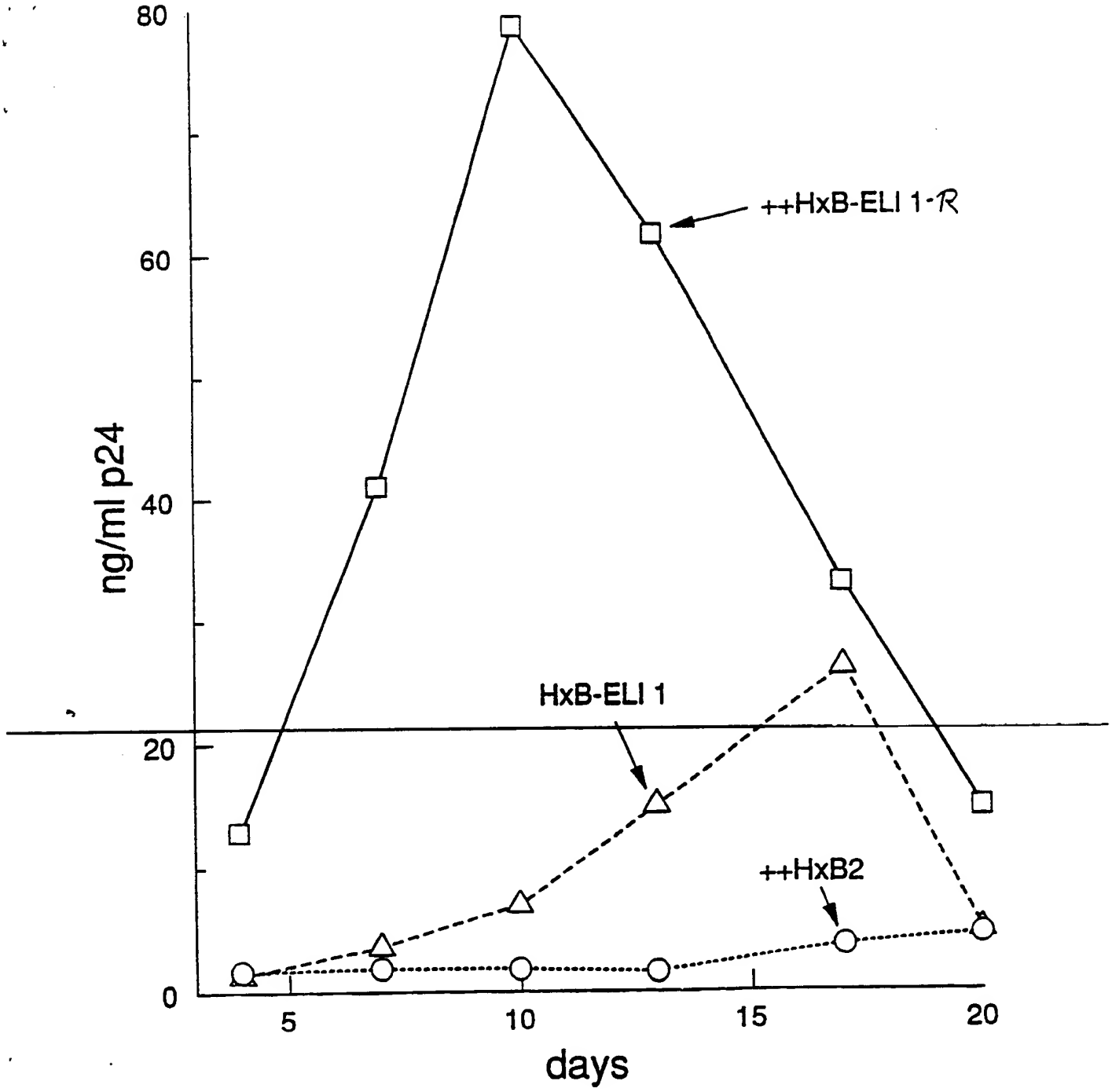

FIGURE 8

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/04333

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12Q 1/68; C12N 15/00

U.S.Cl: 435/6, 172.3, 320.1, 240.2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.Cl	435/6, 172.3, 320.1, 240.2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

U.S. Patents and Chemical Abstracts

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Proceedings National Academy of Science, Vol. 86, issued February 1989, Niederman et al. "Human Immunodeficiency Virus Type 1 Negative Factor is a Transcriptional Silencer," pages 1128-1132, see entire document.	1-13
X	Science, Vol. 246, issued 22 December 1989, Cheng-Mayer etal. "Differential Effects of nef of HIV Replication: Implications for Viral Parthenogenesis in the Host," pages 1629-1632. See entire document.	1-13

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29 October 1991	25 NOV 1991
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Deborah Crouch Deborah Crouch, Ph.D.

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	Nature, Vol. 316, issued 18 July 1985, A. Fisher et al., "A Molecular Clone HTLV III with Biological Activity," pages 262-265, see entire document.	1-13
X	Science, Vol. 247, issued 02 February 1990, J.M. McCune et al., Suppression of HIV Infection in AZT- Treated Scidhu Mice", pages 564-566, see entire document.	14-23

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers ...., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequantly, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	US. A, 4,795,739 (Lifson et al) 03 January 1989, especially col. 3, 4, 15, 22-24 and the claims.	24-27